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STUDIES OF CELLULAR DEFENSE AGAINST INFECTION: THE INTERACTION OF INFLUENZA VIRUS WITH PHAGOCYTIC CELLS AND ITS EFFECT ON PHAGOCYTOSIS

Annual Report

by

William D. Sawyer, M.D.
Department of Microbiology
The Johns Hopkins University School of Medicine

August, 1966

U.S. Army Medical Research and Development Command

Contract DA-49-193-MD 2599
with
The Johns Hopkins University School of Medicine

STUDIES OF CELLULAR DEFENSE AGAINST INFECTION: THE INTERACTION OF INFLUENZA VIRUS WITH PHAGOCYTIC CELLS AND ITS EFFECT ON PHAGOCYTOSIS

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FOREWARD

This work was conducted under contract DA-49-193-MD 2599 (Project No.: 1C622401A096, Task No.: 1C622401A096-01) during the period 1 July 1965 to 30 June 1966. The technical assistance of Mrs. Caroline Mangeniello and Miss Carolyn Scott is gratefully acknowledged.

In conducting the research described in this report, the investigators adhered to the "Principles of Laboratory Animal Care" as established by the National Society for Medical Research.

The information in this report has not been cleared for release to the general public.

The findings in this report are not to be construed as an official Department of the Army position, unless so designated by other authorized documents.

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SUMMARY

These studies are directed toward defining pathogenic mechanisms in combined infection by viruses and bacteria, especially acute bacterial respiratory infection complicating influenza. Of particular interest is the effect of virus upon phagocytic cells. The conclusions from the work of the past year are:

Both influenza A and B viruses rapidly attached to exudative leukocytes of mice, rats, and guinea pigs. At physiological temperatures the WS strain of influenza A virus eluted from all of these cells except guinea pig macrophages; these macrophages ingested the virus. Both the NWS strain of influenza A and influenza B viruses were ingested by PMN and by macrophages of all three species. Once inside leukocytes, influenza virus was rapidly destroyed.

Interaction with influenza virus reduced the function of phagocytic cells. The antiphagocytic activity was exerted upon exudative polymorphonuclear and mononuclear phagocytes and upon alveolar macrophages, and the extent of inhibition depended upon the quantity of virus and the duration of virus-cell interaction. There were, however, major differences between species; the phagocytic activity of guinea pig and mouse leukocytes was reduced by virus, but that of rat cells was unaffected.

Influenza A virus had no effect upon the phagocytic activity of blood leukocytes of guinea pigs. Following incubation with influenza A virus, human blood leukocytes also ingested pneumococci normally.

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TABLES

FIGURES

This is the second Annual Report under Contract DA-49-193-MD 2599. These studies are directed toward defining pathogenic mechanisms in combined viral and bacterial infections, particularly the effect of the virus component upon the phagocytic defenses of the host. The 1965 Report (1) described the results of *in vitro* studies of the interaction of influenza virus with leukocytes of mice, rats, and guinea pigs, and its effect on the phagocytosis of pneumococci. Briefly, the results indicated that influenza A virus attached rapidly to both polymorphonuclear leukocytes (PMN) and macrophages obtained from peritoneal exudates in all three species. At physiological temperatures the subsequent fate of the virus differed both with cell type and species, i.e., virus eluted from PMN of all three species and from mouse and rat macrophages, but entered guinea pig macrophages. The interaction with virus reduced the ability of guinea pig leukocytes, both PMN and macrophages, to phagocyte pneumococci *in vitro*, but had no effect on the phagocytic function of rat PMN. During the past year these studies have been continued and extended to: (a) the interaction of influenza B virus with leukocytes *in vitro*, (b) the effect of influenza viruses on the phagocytic activity of alveolar and blood leukocytes, (c) further definition of the antiphagocytic effect of virus on exudative leukocytes, and (d) the effect of *in vivo* virus-leukocyte interaction on phagocytic function.

Materials and methods:

Three different influenza viruses were employed: Type A - WS strain, Type A - NWS strain (neuraminidase deficient mutant of WS), and Type B - LEE strain. Stocks of allantoic fluid were prepared in 9 to 11 day embryonated eggs, did not contain antibiotics, were negative upon culture in thioglycolate broth, and were stored at -70°C; normal allantoic fluid (NAF) was obtained from uninoculated eggs, and infected allantoic fluid was harvested 44 to 48 hours after inoculation of the embryo with virus. The normal and infected fluids were similar in hydrogen ion (pH = 7.3), sodium (285 meq/l), potassium (10 meq/l), and calcium (2 meq/l) concentration. The virus content of the stocks was: WS - 2048 to 4096 hemagglutinin (HA) units and $10^{8.0}$ to $10^{9.0}$ egg infections dose 50% (EID_{50}); NWS - 256 to 512 HA units and $10^{6.0}$ to $10^{7.0}$ EID_{50}; and LEE - 512 to 2048 HA units and $10^{6.0}$ to $10^{7.0}$ EID_{50}.

Concentrated virus was prepared by centrifugation of infected allantoic fluid (56,000 x G for 90 minutes) and resuspension of the centrifugate in supernatant fluid to one-tenth of the original volume.

WS virus was separated from allantoic fluid by adsorption on and elution from chicken red cells. Five ml of allantoic fluid were incubated with the cells (5 ml of a 10% suspension, separated from diluent) for 30 minutes at 4°C and then centrifuged in the cold. The cells were incubated at 37°C for 60 minutes in 5 ml of Hank's

balanced salt solution with bicarbonate (HBSS). The eluate, collected after centrifugation, contained 256 to 512 HAu/ml. Although some virus was demonstrable in eggs (approximately $10^{6.0}$ EID_{50}/ml), the amount of virus present in the first supernate (absorbed allantoic fluid) was insufficient to agglutinate chicken erythrocytes. For control purposes NAF was treated in a like manner.

WS virus was also separated from allantoic fluid by centrifugation, 100,000 x G for 60 minutes. The centrifugate was resuspended in HBSS to the original volume. The supernate was centrifuged two additional times. The centrifugate contained 4096 HAu/ml, and the supernate contained insufficient virus to agglutinate chicken erythrocytes (egg inoculation indicated approximately $10^{5.0}$ EID_{50}/ ml).

Viral hemagglutinin and egg infectivity were determined by standard methods as previously described (1).

Neuraminidase (receptor-destroying enzyme, RDE) was obtained from Microbiological Associates, Silver Spring, Md.

Animals were: Mice, 18-22 gm female, MBR/ICR strain, obtained from Maryland Breeders for Research, Burtonsville, Md.; Rats, 180-200 gm male, CD strain, obtained from Charles River Breeding Laboratories, Inc., Brookline, Mass.; and Guinea pigs, 300-400 gm male, albino, obtained from John C. Landis, Hagerstown, Md.

Exudate leukocytes were obtained as previously described (1).

Alveolar macrophages were obtained from rats and guinea pigs by washing the cells from the excised lungs of normal animals* (3) with

* Animals were killed with pentobarbital.

modified Hank's solution (2) containing 0.1% bovine serum albumin and 0.2% glucose (HBG). More than 90% of the cells were mononuclear.

Blood leukocytes of guinea pigs were collected from heparinized blood obtained by cardiac puncture. After centrifugation, the buffy coat was harvested, and the cells were washed three times in HBG. Human blood leukocytes were harvested from venous blood after sedimentation of red cells. Blood (100 ml) was drawn into heparinized plastic syringes, mixed with 25 ml of 6% dextran containing 750 mg of glucose and 1 mg of heparin, and allowed to stand for 60 minutes. The upper, fluid phase was aspirated and centrifuged at 250 x G for 5 minutes in the cold. The supernate was discarded, and the leukocytes were washed once with HBG.

Macrophage cultures were prepared in milk dilution bottles. Several media (Grand Island Biological Co.) were employed: 199, lactalbumin hydrolyzate in Earle's salt (ELH), and Eagle's basal medium (BME); all contained 20% calf serum and 200 units of penicillin and 200 µg streptomycin per ml. The inoculum was 2×10^7 washed macrophages in 15 ml of medium. The bottles were incubated in 5% CO_2 at 37°C. The cultures in ELH and in BME appeared to form better sheets and remained attached longer than those in medium 199.

Serum was harvested from blood which had been allowed to clot for 6 hours at 4°C and was stored at -70°C. Guinea pig serum contained heat labile inhibitors of NWS and LEE viruses; inhibitors of WS virus were not detected. Rat serum did not contain inhibitors of the viruses employed.

Maximally encapsulated type I pneumococci were grown and prepared as previously described (1).

Phagocytic function in vitro was determined employing the concentrated test system previously described (1). Briefly, in this test 2.5×10^8 packed cells were mixed with 0.1 ml of homologous normal serum and 4×10^9 pneumococci contained in 0.025 ml. Of this mixture 0.06 ml was spread over a 2 x 2 cm area of a clean glass slide and over a similar area of moistened filter paper (A.S. Aloe Co., St. Louis, Mo.). After 30 minutes incubation at 37°C in a petri dish lined with moistened filter paper, the cells were recovered; smears were prepared, stained with methylene blue, and examined. The results were expressed as the per cent of 400 cells containing pneumococci. The results in Table I summarize tests performed with guinea pig

Table I

Phagocytic Activity of Guinea Pig PMN after Incubation with Normal Allantoic Fluid - 30% Guinea Pig Serum

Test Surface	No. of Tests	Mean % Phagocytosis	Std. Dev.	Mean Difference of Duplicates	Std. Dev.
Glass	24	20	8	2	2
Paper	21	58	13	4	3

peritoneal exudate PMN that had been incubated with normal allantoic fluid - 30% normal guinea pig serum for 2 hours at 37°C. These tests were performed over a 2 year period, and the large standard deviations

reflect the variation in results observed in tests done at widely-spaced intervals. Inspection of the data indicated, however, that multiple tests performed the same day (or at daily intervals) were in close agreement. The high reproducibility was confirmed by mathematical analysis, i.e., the standard deviation of the difference between duplicates was small.* The 95% confidence level was attained by differences between tests of 5% and 9% on glass and paper respectively. Therefore, the results of tests performed upon cells after incubation with a test material were always compared with results of simultaneous tests upon cells treated with a suitable control substance, e.g., cells incubated with infected allantoic fluid and cells incubated with normal allantoic fluid. The results then were expressed as the per cent of the value in the control test.

Results:

Detailed evidence was presented last year (1) indicating that influenza A and B viruses attached to exudate leukocytes of mice, rats, and guinea pigs. At physiological temperatures the fate of adsorbed virus varied, however. WS virus rapidly eluted from guinea pig PMN and from both PMN and macrophages of mice and rats, but was quickly ingested and altered by guinea pig macrophages. The ultimate fate of virus within macrophages was uncertain. No evidence of virus

* Although the number of duplicate tests was small, similar reproducibility was achieved with guinea pig macrophages and with rat PMN and macrophages.

replication was found in experiments in which the macrophages ingested virus prior to establishment of a macrophage culture. In contrast, when an established macrophage culture was exposed to virus, a small amount of virus, e.g., 10^6 EID_{50}, was released into the fluid during the first 24 hours of incubation (the cultures had been extensively washed after exposure to virus, and the last wash fluid found free of virus). The possibility remained that the small amount of virus released into the culture fluid was virus that either eluted subsequent to the wash or was associated with cells that detached after washing, or both. That this possibility did account for the virus recovered was demonstrated in an experiment in which a macrophage culture was exposed to $10^{8.0}$ EID_{50} of WS virus for 1 hour at 37°C, washed 5 times with 20 ml of buffered saline, and incubated with tissue culture medium; the last wash contained less than 10^2 EID_{50}/ml of virus. The virus content of the medium was $10^{6.7}$ and $10^{5.2}$ EID_{50} after 2 and 24 hrs of incubation respectively.* These results, together with those presented last year, indicated that the virus entering macrophages was destroyed.

Preliminary observations, reported last year (1), raised the possibility that the entry of WS virus into guinea pig peritoneal exudate macrophages was facilitated by the presence of a component(s)

* The decrease in virus content of the medium between 2 and 24 hours was consistent with the decline of infectious virus during incubation in medium alone.

of normal serum. Additional studies during the past year have indicated, however, that guinea pig macrophages ingest and destroy equally well virus suspended in normal guinea pig serum, heated (56°C for 30 minutes) guinea pig serum, and non-serum containing solutions.

The interaction of NWS virus with leukocytes differed from that of WS virus. As described last year (1), after attachment to cells, the neuraminidase-deficient NWS virus did not elute and was ingested by leukocytes (mixed cell types) of mice, rats, and guinea pigs. Subsequent studies have indicated that the NWS virus is retained by both PMN and macrophages of all 3 species, Table II. Different numbers of cells from PMN-rich and

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Table II

Reaction of NWS Virus with Mouse, Rat, and Guinea Pig Leukocytes - 37°C for 30 Min.

	No. of Cells ($\times 10^7$)	HA Recovered/HA Added* PMN-Rich Exudate	HA Recovered/HA Added* Macrophage-Rich Exudate
Mouse	2	0.500	0.500
	10	0.062	0.062
Rat	2	0.375	0.508
	5	---	0.162
	10	0.093	0.081
Guinea Pig	2	0.125	0.062
	10	0.016	0.016

* 512 HA units.

macrophage-rich exudates were incubated with NWS virus for 30 minutes at 37°C, and the unattached HA was estimated. The amount of HA removed from the fluid phase was related to the number of leukocytes and was similar with PMN-rich (approximately 80% PMN) and with macrophage-rich (approximately 80%) cell populations. These results were in sharp contrast to those obtained with WS virus and guinea pig leukocytes (see 1965 Annual Report) in which the quantity of virus taken up by leukocytes was directly related to the number of macrophages present, but unrelated to the number of PMN.

In experiments with cultures of macrophages from mice, rats, and guinea pigs, NWS virus did not replicate.

Although the LEE strain of influenza B possesses neuraminidase, the interaction of this virus with rat and guinea pig leukocytes resembled that of the NWS strain of influenza A rather than that of the WS strain. Virus was eliminated from the fluid phase by both PMN and macrophages of rats and guinea pigs, Table III. With both rat and guinea pig cells uptake of virus was related to total cell number and was not restricted to either cell type. Although the differences were small, the uptake of virus by guinea pig leukocytes consistently exceeded that by rat cells; the difference was most apparent with macrophage-rich exudates. Penetration of the cells by virus was indicated by the failure of exogenous RDE to free significant amounts of virus from the cells. Replication of LEE virus was not demonstrated in guinea pig macrophage cultures.

Table III

Reaction of LEE Virus with Rat and Guinea Pig Leukocytes - 37°C for 60 Min.

	No. of Cells ($\times 10^7$)	HA Recovered/HA Added*	
		PMN-Rich Exudate	Macrophage-Rich Exudate
Rat	1	0.250	0.500
	2	0.125	0.250
	4	0.062	0.125
	6	0.031	0.062
Guinea Pig	1	0.250	0.250
	2	0.125	0.062
	4	0.062	0.031
	6	0.031	0.016

* 256 HA units.

During experiments to determine the effect of WS virus upon the phagocytic activity of rat and guinea pig alveolar macrophages, the uptake of virus by the macrophage preparations was measured in a single experiment for each species, Table IV. The results were consistent with those obtained with macrophages from peritoneal exudates, i.e., uptake by guinea pig cells but not by rat cells. Because of the difficulties in obtaining large numbers of alveolar macrophages, detailed studies of attachment and elution or penetration have not been attempted.

Table IV

Reaction of WS Virus with Rat and Guinea Pig Alveolar Macrophages - 37°C for 120 Min.

Species	No. of Cells ($\times 10^7$)	HA units Added	HA units Recovered
Rat	6.5	512	512
Guinea Pig	15.0	600	<5

Also in conjunction with phagocytic studies, the uptake of WS virus by blood leukocytes of guinea pigs and man was demonstrated, Table V. Because the cell populations were of a mixed nature,* i.e.,

Table V

Reaction of WS Virus with Guinea Pig and Human Blood Leukocytes - 37°C for 120 Min.

Species	No. of Cells ($\times 10^7$)	HA units Added	HA units Recovered
Guinea Pig	12.5	2048	256
Man	15.0	4096	1024

PMN, lymphocytes, and monocytes, the cell type(s) responsible for virus uptake was not determined. The amount of virus removed by the

* Mean differential counts were:

	PMN	Lymphocytes	Monocytes
Guinea Pig	47%	44%	9%
Man	70%	20%	10%

guinea pig blood leukocytes was similar to that eliminated by the same number of cells from a PMN-rich peritoneal exudate, but was less than that taken up by cells from a macrophage-rich exudate.

The previous annual report (1) described limited studies of the effect of the in vitro interaction of influenza virus on the phagocytic activity of leukocytes from peritoneal exudates of rats and guinea pigs. The in vitro virus-cell interaction depressed phagocytosis of type I pneumococci by PMN and macrophages of guinea pigs but not by PMN of rats. Reduced phagocytic activity was related to the duration of virus-cell interaction and was apparent only in phagocytic tests employing highly concentrated mixtures of cells and pneumococci. Other cell functions, e.g., motility and ability to exclude eosin Y, were adversely affected by virus-cell interactions that impaired phagocytic activity. Because this report includes an extension of some of these studies, no attempt will be made to separate the portions of an investigation that were performed in one year or the other.

The phagocytic activity of guinea pig leukocytes was reduced after incubation with WS, NWS, and LEE viruses, Fig. 1. Because the quantity of virus influenced the extent of reduction in activity (see below) and the preparations were not normalized in these experiments, the antiphagocytic action of the different viruses cannot be compared directly. Although the cells were incubated with virus (or NAF) and 30% by volume of normal guinea pig serum in these experiments, normal

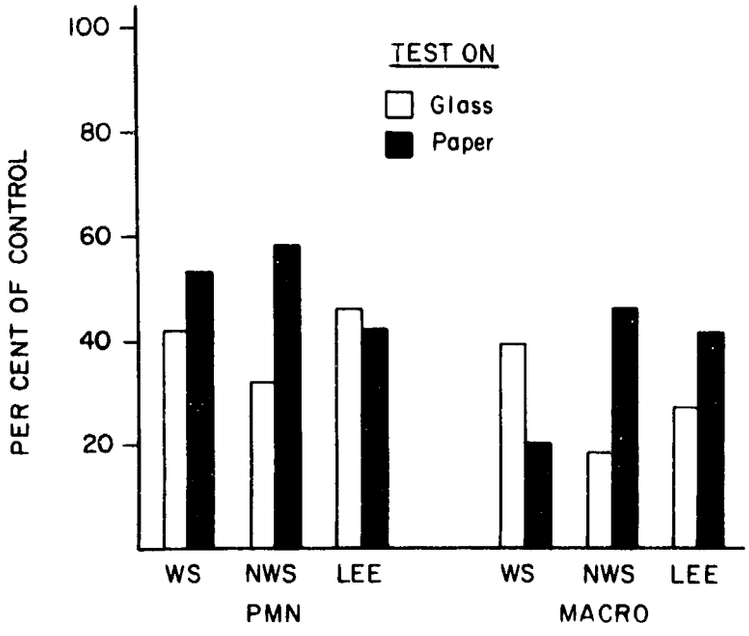

Fig. 1. The effect of influenza virus upon the phagocytic activity of leukocytes of guinea pig exudate. PMN and macrophages were incubated for 120 and 60 minutes respectively at 37°C with virus (or NAF in the control) and 30% of guinea pig serum prior to the phagocytic test. Means are shown.

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serum was not essential for the antiphagocytic action of the virus*, i.e., the phagocytic activity of guinea pig PMN was reduced after incubation of the cells in WS-infected allantoic fluid and in infected fluid with heated serum (56°C for 30 minutes), Tyrode's solution, and saline (Fig. 2).

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* An essential role of serum was suggested in last year's report.

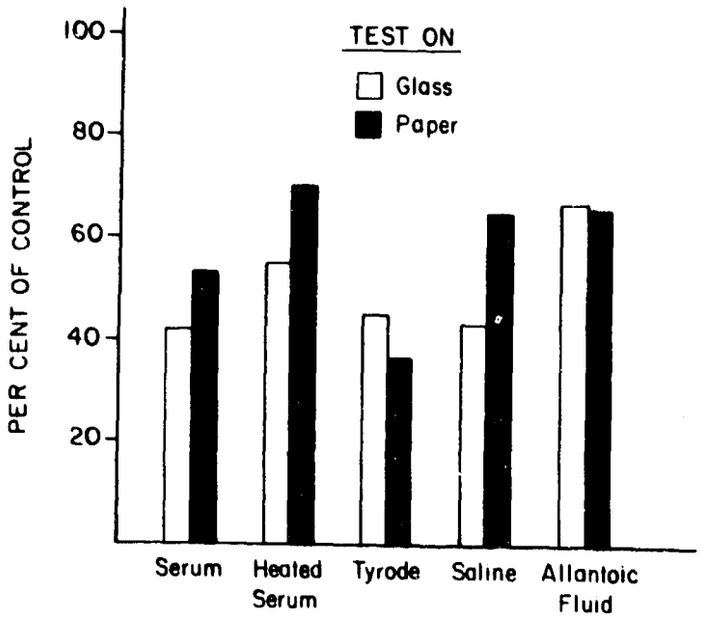

Fig. 2. The effect of serum and buffers upon the antiphagocytic action of WS virus upon guinea pig exudate PMN. Cells were incubated with virus (or NAF in the control) together with 30% of the test material for 120 min at 37°C prior to the phagocytic test.

The quantity of virus affected the extent of depression of phagocytic activity (Fig. 3). The previous report (1) described the relationship between the duration of virus-cell incubation and the reduction in phagocytic activity.

In the experiments described thus far, infected allantoic fluid was compared with normal allantoic fluid. When allantoic fluid from infected embryos was treated (absorbed or centrifuged) such that

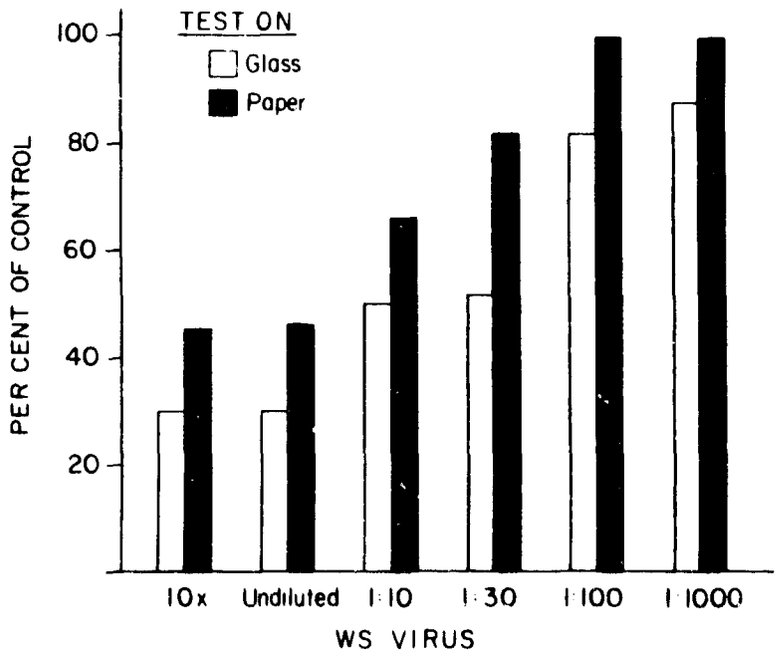

Fig. 3. The effect of different amounts of WS virus upon the phagocytic function of guinea pig exudate PMN. Virus was diluted with normal allantoic fluid and incubated, together with 30% of guinea pig serum, with the cells for 120 min at 37°C prior to the phagocytic test. The control was NAF - 30% serum.

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most of the virus was removed, it was without effect on the phagocytic activity of guinea pig PMN (Fig. 4). The recovered virus (eluate or centrifugate suspended in HBSS) reproduced the effect of the infected allantoic fluid (Fig. 4). Because the two methods of removing virus were so different in mechanism, it is unlikely that some component other than virus accounted for the antiphagocytic action of infected allantoic fluid.

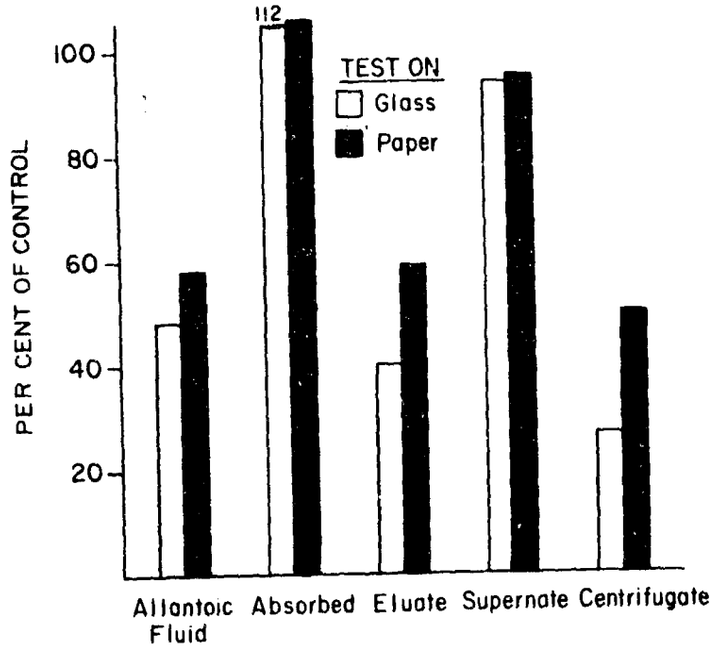

Fig. 4. Association of antiphagocytic action of WS allantoic fluid with the virus component. Prior to the phagocytic test guinea pig exudative PMN were incubated for 120 min at 37°C in the test material and 30% of guinea pig serum. Controls were NAF, treated in the same way, with 30% serum.

Leukocytes from rat exudates had normal phagocytic capacity after in vitro interaction with the 3 strains of influenza virus (Fig. 5). Identical results were obtained with 10-fold concentrated virus preparations.

With mouse exudative PMN, control values were low, e.g., 5% phagocytosis on glass and 25 to 30% on paper. Interaction with WS and NWS viruses, however, significantly reduced phagocytic activity,

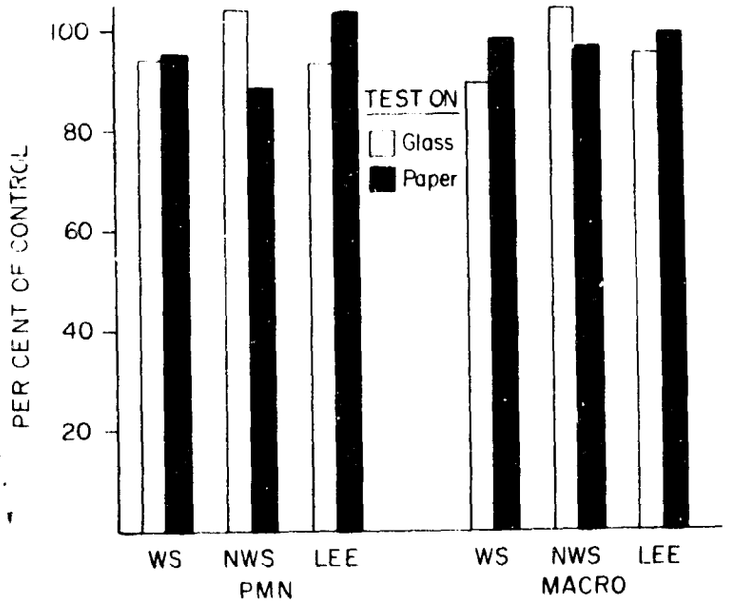

Fig. 5. The effect of influenza virus upon the phagocytic activity of leukocytes of rat exudate. Prior to the phagocytic test PMN and macrophages were incubated for 120 and 60 min respectively at 37°C with the virus (or NAF in the control) and 30% of rat serum. Means are shown.

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i.e., to 43% and 29% respectively of the NAF control in the tests on filter paper. The effect of virus on the phagocytic activity of mouse macrophages has not been determined.

Interaction with WS virus in vitro affected the phagocytic activity of rat and guinea pig alveolar macrophages in a way similar to peritoneal macrophages, i.e., the phagocytic activity of guinea pig alveolar macrophages was reduced, but that of rat alveolar macrophages was unaffected (Fig. 6).

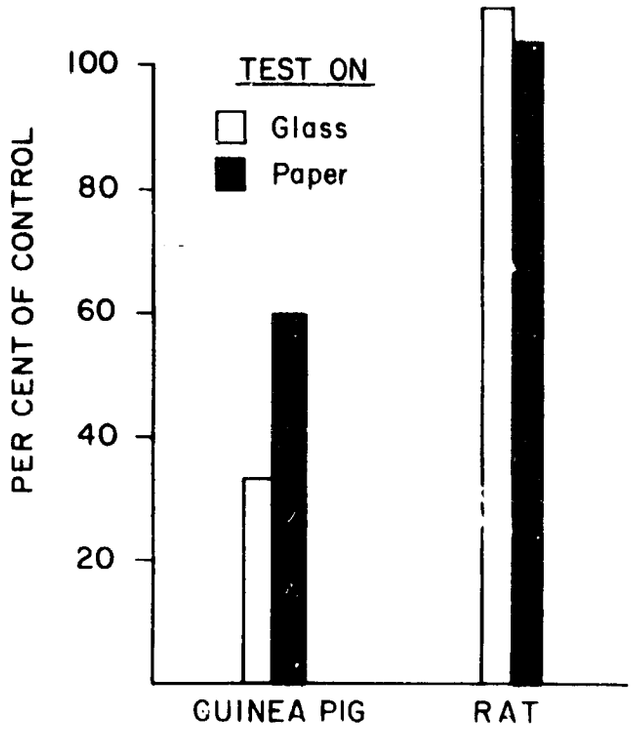

Fig. 6. The effect of WS virus upon the phagocytic activity of alveolar macrophages of guinea pigs and rats. Cells were incubated at 37°C for 60 min with virus (or NAF in the control) and 30% of homologous serum prior to the phagocytic test.

Guinea pig blood PMN differed markedly from exudative PMN in susceptibility to the antiphagocytic action of WS virus. Under conditions of virus-cell interaction that consistently decreased phagocytosis by exudative PMN, blood PMN retained normal phagocytic

activity (Table VI). The phagocytic activity of human blood PMN* was also normal after incubation with WS virus (Table VI).

Table VI

Effect of WS Virus upon the Phagocytic Activity of Blood PMN

Cells Incubated with*	Mean Per Cent Phagocytosis			
	Guinea Pig		Human	
	Glass**	Paper**	Glass	Paper
NAF	22	52	16	50
WS	21	56	18	52

* Cells were incubated with normal allantoic fluid (NAF) or with virus for 120 min at 37°C prior to the phagocytic test.

** Surface in phagocytic test.

To determine if virus-leukocyte interactions *in vivo* could affect phagocytic function, WS virus, or NAF, was injected intraperitoneally into animals bearing peritoneal exudates of the desired predominant cell type. After a period identical to that employed for *in vitro* interactions (60 minutes with macrophages, 120 minutes with PMN), the animals were sacrificed, and the cells were harvested and tested for phagocytic activity *in vitro*. Initially,

* Tests were performed upon leukocytes from 2 individuals; one had no hemagglutination inhibiting antibody, and the other had a titer of 1:40 against WS virus. The results were similar with both sets of cells.

the amounts of virus employed (2048 to 4096 HA units) were similar to those used *in vitro*; the phagocytic activity of guinea pig leukocytes was unaffected. Because the exudate in the animal is constantly changing, the injected virus might not have reached all cells, especially cells entering the exudate during the "incubation" period. To overcome this variable, larger amounts of virus (16,000 to 41,000 HA units) and NAF were employed, and injections were made at intervals (every 15 to 30 minutes) throughout the period of virus-cell contact. The results were similar to those obtained with virus-cell interaction *in vitro*, i.e., the phagocytic activity of guinea pig PMN and macrophages was reduced, but that of rat leukocytes remained normal (Fig. 7).

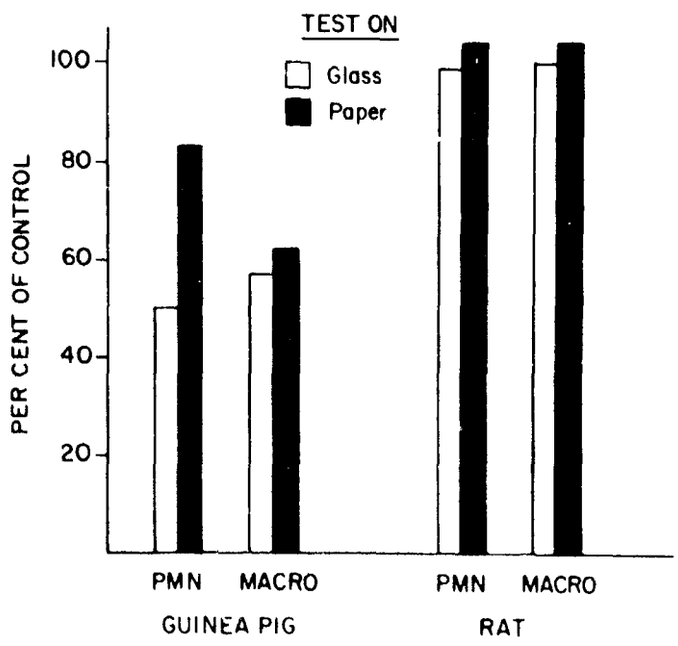

Fig. 7. Effect of interaction *in vivo* with WS virus upon the phagocytic activity of exudate leukocytes of guinea pigs and rats. See text. Means are shown.

Exudative PMN and macrophages from rats receiving intraperitoneal NAF adsorbed from 8 to 32 times more virus at 4°C than did cells from animals injected with WS virus. These results were consistent with receptor destruction by viral neuraminidase (for *in vitro* studies see 1965 Annual Report) and, therefore, indicated that effective virus-cell contact had occurred in the *in vivo* experiments.

Discussion:

Table VII summarizes the net results of the interaction at 37°C of influenza A and B viruses with the leukocytes from peritoneal exudates of mice, rats, and guinea pigs. The cells have abundant receptors for the viruses, and attachment occurs rapidly. The neuraminidase of WS virus causes elution from all the cell types except guinea pig macrophages. Even with these cells repeated attachment and elution of individual particles may occur, but the end result is attachment and entry into the cell; once within the cell the virus is destroyed. The mechanism is unknown whereby the guinea pig macrophage ingests attached virus while the other cell types fail to do so. Presumably the explanation lies in the rates of the competing events, i.e., enzymatic elution and the phagocytic activity of the cell; data on the kinetics of these events are lacking. It is clear, however, that elimination of the elution process, e.g., the neuraminidase-deficient NWS strain, leads to ingestion of virus by both cell types of the 3 species studied. Although LEE virus possesses neuraminidase, it is ingested by all the cell

Table VII

Reaction at 37°C of Influenza Viruses with Exudate Leukocytes of the Mouse, Rat, and Guinea Pig

Cells		WS			NWS			LEE		
		Attach	Elute	Pene-trate	Attach	Elute	Pene-trate	Attach	Elute	Pene-trate
Mouse	PMN	+	+	-	+	-	+			
	Macro	+	+	-	+	-	+			
Rat	PMN	+	+	-	+	-	+	+	-	+
	Macro	+	+	-	+	-	+	+	-	+
Guinea Pig	PMN	+	+	-	+	-	+	+	-	+
	Macro	+	-	+	+	-	+	+	-	+

types. The reasons for this surprising finding are unknown. Again, differences in the kinetics of elution and ingestion presumably account for the results. It has been suggested that the amount of receptor material that must be destroyed prior to elution is greater for LEE virus than for WS virus (4). If this suggestion is true for leukocytes, WS may elute from most cells before the occurrence of an irreversible part of the ingestion process, but LEE remains attached for a longer time and is ingested.

Whereas interaction with influenza virus both in vitro and in vivo reduces the phagocytic activity of certain peritoneal leukocytes, the virus does not alter this function of other leukocytes (Table VIII). The outcome of the virus-cell interaction is not correlated with the effect on phagocytosis; e.g., virus attachment and elution may or may not affect phagocytosis, and virus penetration of the cell may or may not be associated with altered function. Furthermore, the action of viral neuraminidase can not account for the effect on cell function, i.e., neuraminidase-deficient and neuraminidase-rich viruses have similar effects. The only apparent correlation is the effect of the viruses on the phagocytic activity of guinea pig cells, and presumably upon mouse cells as well, and the lack of effect on rat leukocytes. Knowledge of the comparitive biology of leukocytes is limited, and further studies are needed to explain the differences observed here between the species. The work of Fisher and Ginsberg (5, 6) may provide a good starting point. If influenza virus decreases

Table VIII

Interaction *in vitro* of Influenza Viruses with Exudate Leukocytes of the Mouse, Rat, and Guinea Pig at 37°C and Its Effect on Phagocytic Activity

Cells		WS		NWS		LEE	
		Attached Virus	Phago-cytosis	Attached Virus	Phago-cytosis	Attached Virus	Phago-cytosis
Mouse	PMN	Elutes	Reduced	Pene-trates	Reduced		
Rat	PMN	Elutes	Normal	Pene-trates	Normal	Pene-trates	Normal
	Macro	Elutes	Normal	Pene-trates	Normal	Pene-trates	Normal
Guinea Pig	PMN	Elutes	Reduced	Pene-trates	Reduced	Pene-trates	Reduced
	Macro	Pene-trates	Reduced	Pene-trates	Reduced	Pene-trates	Reduced

phagocytosis by inhibiting glycolysis as they suggest, significant species differences should be demonstrable in the effect of virus on leukocyte glycolysis.

Alveolar macrophages differ from exudate cells in several significant ways (7, 8), particularly with regard to adaptation to an aerobic environment; i.e., the energy for phagocytosis by alveolar macrophages is derived both from glycolysis and oxidative metabolism (8). Despite these differences, influenza virus affects the phagocytic activity of alveolar cells as it does exudative macrophages of the same species, i.e., the activity of guinea pig cells is decreased, but that of rat cells remains normal. The killing of Staphylococcus albus deposited in the depths of the lungs of mice has been attributed primarily to the phagocytic activity of alveolar macrophages (9), and, interestingly, such killing was shown to be reduced on the fifth day of infection of the respiratory tract by influenza virus (10).

Guinea pig blood PMN appear to resist the antiphagocytic action of influenza virus. Although these results conflict with those obtained with guinea pig leukocytes collected from other sites, differences between blood and exudate leukocytes are known in other systems. For example, rat blood leukocytes continue to phagocyte bacteria in hyperosmolar solutions that inhibit phagocytosis by exudate PMN (11). In a different model, the release of leukocytic pyrogen by rabbit PMN, exudate cells spontaneously release pyrogen

during incubation in isotonic sodium chloride, but blood PMN do not (12); blood cells release pyrogen spontaneously only after activation, i.e., after incubation with a material (activator) found in exudate fluid (12). Thus, the present studies with influenza virus appear to add another item to the list of differences between blood and exudate leukocytes. Such differences, however, complicate determination of the effect of virus upon human leukocytes. Blood cells are most readily examined, but results such as those in the present study need not reflect the effect of influenza virus upon leukocytes outside the blood stream, those leukocytes that are of primary importance in the defense of the respiratory tract against acute bacterial infection (13).

The present investigations have not defined the role of the antiphagocytic action of influenza virus in the pathogenesis of combined influenzal and bacterial infection. They do, however, confirm that the phenomenon is of potential significance and provide a basis for investigation in intact animal models. In particular, species differences in susceptibility to viral antiphagocytic action, rat versus guinea pig, may provide a valuable tool. If comparable lesions can be produced in the two species with influenza virus, a significant antiphagocytic effect of virus should result in impaired clearance of bacteria from guinea pig lungs compared to rat lungs.

Conclusions:

Both influenza A and B viruses rapidly attached to exudative leukocytes of mice, rats, and guinea pigs. At physiological tempera-

tures the WS strain of influenza A virus eluted from all of these cells except guinea pig macrophages; these macrophages ingested the virus. Both the NWS strain of influenza A and influenza B viruses were ingested by PMN and by macrophages of all three species. Once inside leukocytes, influenza virus was rapidly destroyed.

Interaction with influenza virus reduced the function of phagocytic cells. The antiphagocytic activity was exerted upon exudative polymorphonuclear and mononuclear phagocytes and upon alveolar macrophages, and the extent of inhibition depended upon the quantity of virus and the duration of virus-cell interaction. There were, however, major differences between species; the phagocytic activity of guinea pig and mouse leukocytes was reduced by virus, but that of rat cells was unaffected.

Influenza A virus had no effect upon the phagocytic activity of blood leukocytes of guinea pigs. Following incubation with influenza A virus human blood leukocytes also ingested pneumococci normally.

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13. ABSTRACT

These studies are directed toward defining pathogenic mechanisms in combined infection by viruses and bacteria, especially acute bacterial respiratory infection complicating influenza. Of particular interest is the effect of virus upon phagocytic cells. The conclusions from the work of the past year are:

Both influenza A and B viruses rapidly attached to exudative leukocytes of mice, rats, and guinea pigs. At physiological temperatures the WS strain of influenza A virus eluted from all of these cells except guinea pig macrophages; these macrophages ingested the virus. Both the NWS strain of influenza A and influenza B viruses were ingested by PMN and by macrophages of all three species. Once inside leukocytes, influenza virus was rapidly destroyed.

Interaction with influenza virus reduced the function of phagocytic cells. The antiphagocytic activity was exerted upon exudative polymorphonuclear and mononuclear phagocytes and upon alveolar macrophages, and the extent of inhibition depended upon the quantity of virus and the duration of virus-cell interaction. There were, however, major differences between species; the phagocytic activity of guinea pig and mouse leukocytes was reduced by virus, but that of rat cells was unaffected.

Influenza A virus had no effect upon the phagocytic activity of blood

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